QTL analysis and comparative genomics of herbage quality traits in perennial ryegrass (Lolium perenne L.)

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Abstract

Genetic control of herbage quality variation was assessed through the use of the molecular marker-based reference genetic map of perennial ryegrass (Lolium perenne L.). The restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and genomic DNA-derived simple sequence repeat (SSR)-based framework marker set was enhanced with RFLP loci corresponding to genes for key enzymes involved in lignin biosynthesis and fructan metabolism. Quality traits such as crude protein (CP) content, estimated in vivo dry matter digestibility (IVVDMD), neutral detergent fibre (NDF) content, estimated metabolisable energy (EstME) and water soluble carbohydrate (WSC) content were measured by near infra-red reflectance spectroscopy (NIRS) analysis of herbage harvests. Quantitative trait locus (QTL) analysis was performed using single marker regression, simple interval mapping and composite interval mapping approaches, detecting a total of 42 QTLs from six different sampling experiments varying by developmental stage (anthesis or vegetative growth), location or year. Coincident QTLs were detected on linkage groups (LGs) 3, 5 and 7. The region on LG3 was associated with variation for all measured traits across various experimental datasets. The region on LG7 was associated with variation for all traits except CP, and is located in the vicinity of the lignin biosynthesis gene loci xlpomt1 (caffeic acid-*O*-methyltransferase), xlpccr1 (cinnamoyl CoA-reductase) and xlpssrcad2.1 (cinnamyl alcohol dehydrogenase). Comparative genomics analysis of these gene classes with wheat (Triticum aestivum L.) provides evidence for conservation of gene order over evolutionary time and the basis for cross-specific genetic information transfer. The identification of co-location between QTLs and functionally-associated genetic markers is critical for the implementation of marker-assisted selection programs and for linkage disequilibrium studies, which will enable future improvement strategies for perennial ryegrass.

Keywords: *Perennial ryegrass* *Genetic map* *Herbage quality* *Quantitative trait locus* *Functionally-defined gene* *Lignin*

Introduction

The composition of cell walls, particularly the content and cross-linking of lignin, is an important determinant of herbage digestibility (Buxton and Russell 1988), while the biosynthesis of soluble oligosaccharides such as fructans is of key importance for energy provision to the grazing animal (Michell 1973; Jones and Roberts 1991). The genetic control of nutritive value parameters in pasture species has been reviewed (e.g. Ulyatt 1981; Stone 1994; Casler 2001), and genetic variation for specific traits has been established. Digestibility is generally considered to be the most important temperate grass nutritive value trait for either live-weight gain (Wheeler and Corbett 1989) or dairy production (Smith et al. 1997). Deliberate attempts to improve dry matter digestibility (DMD) in forage crop species have led to rates of genetic gain in the range of 1 - 4.7% per annum as a proportion of the initial population means (Casler 2001). Progress in simultaneous improvement of yield and DMD in forage grasses has, however, been variable (Wilkins and Humphreys 2003).

Forage quality may be directly evaluated by feeding trials, but this approach is costly and limited for small quantities of herbage from breeding experiments. Indirect methods of assessment include in vitro digestibility with rumen liquor (Menke et al. 1979; Tilly and Terry 1963), enzymatic digestion (De Boever et al. 1986) and chemical analysis of cellular components (van Soest 1963). The development of near infra-red reflectance spectroscopy (NIRS) analysis for prediction of forage quality has facilitated rapid and non-destructive evaluation of samples from plant breeding programs. NIRS has been used to develop calibrations to predict a wide range of forage quality traits (Marten et al. 1984; Smith and Flinn 1991) including crude protein (CP) content, estimated in vivo dry matter digestibility (IVVDMD), neutral detergent fibre (NDF) content (Smith and Flinn 1991) and water-soluble carbohydrate (WSC) content (Smith and Kearney 2000) in perennial ryegrass. NIRS estimates of DMD and related nutritive value traits have been reported in a range of forage systems (e.g. Carpenter and Casler 1990; Hopkins et al. 1995, Smith et al. 2004).

The reference genetic map for perennial ryegrass based on RFLP, AFLP and SSR loci (Jones et al. 2002a,b) provides the basis for the

genetic dissection of phenotypic traits that vary in the mapping population. QTLs for a number of traits related to vegetative and reproductive morphogenesis, reproductive development and winter hardiness have already been identified (Yamada et al. 2004). The framework marker set, that is dominated by anonymous and non-genic genetic markers, may be selectively enhanced with functionally-associated genetic markers based on expressed sequences (Kurata et al. 1994; Chao et al. 1994; Schneider et al. 1999; Tanksley et al. 1992). The genetic map assignment of loci detected by genes associated with specific biochemical pathways permits evaluation of co-location between such loci and QTLs for putatively correlated traits. A functionally-associated marker-based genetic map of potato (Chen et al. 2001) containing genes involved in carbohydrate metabolism and transport has been used to detect co-locations with QTLs for tuber starch content. Similar studies have been performed with specific functionally-defined genes for traits such as disease resistance, grain quality attributes, secondary metabolite biosynthesis and flowering time across a range of crop species (Faris et al. 1999; Francki et al. 2004; Li et al. 2004; Pflieger et al. 2001; Huh et al. 2001; Lagercrantz et al. 1996). For nutritive quality traits in grass herbage, genes involved in lignin and fructan metabolism provide primary candidates for analysis. Perennial ryegrass cDNAs encoding enzymes involved in lignin biosynthesis (Heath et al. 1998; Heath et al. 2002; Lynch et al. 2002; McInnes et al. 2002) and fructan metabolism (Lidgett et al. 2002; Johnson et al. 2003; Chalmers et al. 2003) have been isolated and characterised. Genetic dissection of herbage quality characters is consequently accessible to both anonymous and functionally-associated marker systems.

Comparative genetic mapping in perennial ryegrass based on heterologous RFLP anchor probes revealed conserved syntenic relationships between the genome of perennial ryegrass and those of other Poaceae species (Jones et al. 2002a). Similarities in genetic map structure were particularly evident with the Triticeae cereals, such that each perennial ryegrass LG showed a predominant correspondence to one of the homoeologous groups of wheat and barley. The development of comparative genomics analysis based on sequence comparison and

ortholocus prediction between Poaceae genomes has become possible through the provision of large expressed sequence tag (EST) collections for several species and draft genome sequences for the grass model species, rice (Goff et al. 2002; Yu et al. 2002). The locations of mapped functionally-defined genes in a species such as perennial ryegrass may be compared to those of putative ortholoci in rice through sequence alignment with map-ordered bacterial artificial chromosome (BAC) clones (Chen et al. 2002). Equivalent ortholocus analysis in wheat may be performed through the mapping of representative ESTs from contigs and singletons to regions based on deletion bins (Endo and Gill 1996; Qi et al. 2003; Sorrells et al. 2003). The grasses of the Poeae tribe, including the Lolium genus, are more closely allied to the cereals of the Triticeae tribe within the Pooideae sub-family of the Poaceae than to the Oryzeae (Soreng and Davis 1998). This close taxonomic affinity suggests that comparative genomics analysis between the Poeae and the Triticeae tribes may prove particularly effective for the identification of common genomic structures, gene orders and orthologous QTL locations.

The aim of this study was to determine the genetic control of herbage quality through the use of data from multiple phenotypic trials, and to identify QTL-linked molecular marker loci suitable for selection experiments. A number of genetically mapped lignin biosynthetic genes have been evaluated for coincidence with QTL-containing regions. Comparative genomics analysis with wheat has been used to explore the genomic distribution and evolution of genes for lignin biosynthesis.

Materials and Methods

Plant materials

The p150/112 reference genetic mapping population was derived from a pair-cross between a multiply heterozygous plant as pollinator and a doubled haploid (DH) as the female parent (Bert et al. 1999; Jones et al. 2002a,b). The cross was generated at the Institute of Grassland and Environmental Research (IGER), Aberystwyth, UK, and clonal replicates of up to 183 progeny individuals and the heterozygous parent were distributed to International Lolium Genome Initiative (ILGI) participant laboratories for genetic and phenotypic analyses. The DH genotype (DH290) did not survive and was consequently not available for phenotypic analysis.

Clonal individual plants were grown in small pots (1/10,000 a), either in glasshouses at the Yamanashi Prefectural Dairy Experiment Station (YPDES), Nagasaka, Japan ((35^{o}49' N, 138^{o}22' E) and the National Agricultural Research Centre for Hokkaido Region (NARCH), Sapporo, Japan (43^{o}00' N, 141^{o}25' E), or in a nursery area outside the glasshouse at NARCH. For the sampling of material at reproductive maturity in the glasshouse, vernalisation was performed during winter by setting the temperature at 7.5 $\pm$ 2.5^{o}C.

Samples were prepared for herbage quality analyses from individual plants at six different times or locations. In 1998 and 1999, samples were taken from plants grown at YPDES with a stubble height of 5 cm on the same June day in each year. The potted plants had previously been cut back at intervals of three weeks duration during the spring. The samples contained leaves with stems. For plants grown at NARCH, the growth stage of the plants (vegetative or reproductive) was considered during sampling. Material was collected at heading time (May or June) for glasshouse-grown plants in 2002 and plants grown in the nursery from April in 2002. The samples were taken from each plant at the individual time of heading at the first cut of the season. Material was collected at the vegetative growth stage on the same late August day in each year for glasshouse-grown plants in both 2001 and 2002. The leafy plants were

again sampled at 5 cm stubble height. Tissue samples were placed in paper bags and dried at 60^{o}C. Dried samples were ground through the 1 mm screen of a cyclone mill.

Near infra-red reflectance spectroscopy analysis

The ground herbage samples were scanned using an NIRSystems Model 5000 scanning monochromator connected to an IBM-compatible personal computer. Infrasoft International (Port Matilda, PA, USA) software was used during NIRS data collection and manipulation. Absorbances were measured, as $\log_{10}$ (1/reflectance) = log (1/R), at 2 nm intervals throughout the near infra-red region (1100-2500 nm). Samples were scanned twice and the spectra were stored as the mean of these 2 samples.

NIRS spectra were transformed by a mathematical treatment designated as 2,5,5,1 (Windham et al. 1989) prior to the development of NIRS equations. The first number in this formula denotes that the second derivative of the $\log_{10}$ (1/R) spectrum was taken, the second denotes the segment gap over which the derivative was calculated, and the third and fourth are the number of data points used during smoothing of the spectrum (Williams 1987). Stepwise multiple linear regression (SMLR) PLS techniques (Shenk and Westerhaus 1991) were then used to develop NIR calibration equations for each constituent from the subset.

Statistical analysis of data

Analysis of variation was performed using GenStat for Windows, 6th Edition (www.vsn-intl.com), to identify significant differences between genotypes and replicate structure for all analysed traits.

QTL analysis

A framework set of genetic markers from the p150/112-based reference map (Jones et al. 2002a), including the majority of the heterologous RFLP loci, was combined with the perennial ryegrass SSR locus data (Jones et al. 2002b) to produce a composite dataset for QTL analysis of the phenotypic data. Following genetic map construction using MAPMAKER 3.0, a sub-set of marker loci was selected to provide even coverage of the

genome with marker intervals of approximately 5 cM, and consensus map distances were subsequently used. Single marker regression (SMR) was initially employed to identify significant variation associated with selected genetic markers. Simple interval mapping (SIM: Lander and Botstein 1989, Haley and Knott 1992) and composite interval mapping (CIM: Zeng, 1994) methods were used to identify and confirm the presence of QTLs. All analyses were performed using the QTL Cartographer 2.0 application (Basten *et al.,* 1994). The maximum log-of-odds (LOD) score of association between the genotype and trait data was calculated for SIM and CIM, and QTL location predictions were accepted for SIM for values greater than a threshold value of 2.5. Permutation analysis (1000 iterations) was used to establish an experiment-wise significance value at the 0.05 confidence level defined as a minimum LOD threshold for each trait in CIM (Churchill and Doerge 1994; Doerge and Churchill 1996). For each form of interval analysis, the maximum LOD value, location of the maximum LOD value on the genetic map, additive marker allele effects and the proportion of phenotypic variance attributable to the QTL were tabulated.

Comparative genomics analysis

Wheat ESTs related to lignin biosynthetic genes from other plant species were identified by sequence annotation using the wEST-SQL database in the GrainGenes resource. The nucleotide sequences were used for TBLASTX analysis (version 2.2.6) through the National Center for Biological Information (NCBI) facility. The chromosomal location of wheat ESTs based on assignment to deletion bins (Qi et al. 2003) were determined using the Mapped Loci query function in Graingenes-SQL (http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi).

Results

Statistical analysis of herbage quality data

For each of the measured traits, significant variation was detected between members of the mapping population ($p<0.001$) for all of the experimental datasets, treated here as replicates. A large proportion of the total variance was explained by the replicate structure. The replicates were based upon on measurement at different stages of development and growth conditions, which increases the relevance of the overall analysis and conclusions, but also has impact on the replicate variance. The glasshouse-grown spring harvest in 2002 was specifically compared with the summer harvest in 2002, to determine the effect of the developmental stage variation on the analysis. The replicate structure was significantly different between the two datasets ($p<0.001$). However, in all cases there was still significant variation explained by the genotypes ($p<0.01$). To assess the replicate nature of the datasets, the two temporal replicates (2001 and 2002) of summer harvests were compared. For the CP, NDF and WSC traits there was significant variation between the replicates ($p<0.001$). For the EstME and IVVDMD traits, significant variation was not detected between the replicates ($p=0.18$ in both cases). In contrast, the two experimental datasets from spring 2002 were analysed together, as a comparative assessment of glasshouse and nursery conditions at the same developmental stage. The replicate structure was again not significantly different for EstME and IVVDMD ($p=0.53$ in both cases), while for the other traits there was significant variation between the replicates ($p<0.01$).

QTL analysis of herbage quality data

Table 1

Figure 1

For each of the traits significant regression was detected between trait and marker data at various positions. No significant association was detected between any of the traits and any marker on linkage group 6. All other

linkage groups displayed significant associations between markers and traits (Table 1, Figure 1). Variable numbers of QTL were identified from the different sampling experiments. The minimum number of QTLs detected from a single dataset were from the summer harvests in 2001 and 2002, with 3 QTLs in each instance, solely for the CP and NDF traits. The maximum number of QTLs detected from single datasets were derived from the spring 2002 nursery-grown harvest and the 1998 harvest. In these instances, 11 QTLs were identified across all traits. However, QTLs for CP and WSC were not detected in the dataset for the nursery-grown spring harvest in 2002.

Crude protein (CP)

A total of 7 QTLs for CP were identified from 5 of the experimental datasets, with the exception of the nursery-grown spring harvest in 2002. Five QTLs failed to show significance with all three analytical methods and should be consequently treated with caution. The QTLs detected on LG1 from the summer 2002 harvest, LG3 from the summer 2001 and 1998 harvests and LG5 from the 1999 harvest were not significantly detected by SIM. However, in all cases there was significant marker-trait association using SMR, and CIM was significant for the LG1 summer 2002 harvest and LG3 summer 2001 harvest QTLs. For the other 1998 and 1999-derived QTLs, maximum LOD values from CIM were not significantly greater that the empirically-set threshold. However, both maximum values exceeded 2.0, and the location of the 1998 harvest QTL was coincident with the equivalent region identified from the spring 2002 and summer 2001 harvests.

Coincident QTLs were identified on LG3 from the datasets of the harvests in spring (glasshouse-grown) 2002, summer 2001 and 1998. The additive effect from the spring harvest was negative, while the effects from the other two harvests were positive (Table 1). The estimated percentage of phenotypic variance explained by the QTLs varied from 6.5%-19.3%, depending on sampling experiment and analytical method. Individual QTLs were detected in a single experimental dataset on four instances (LGs 1, 2, 4 and 5 for harvest years 2002, 2001, 1998 and 1999 respectively).

Estimated in vivo *dry matter digestibility (IVVDMD)*

A total of 8 QTLs for IVVDMD were identified from 4 of the experimental datasets. No significant QTLs were detected from the summer harvests in 2001 and 2002. Five QTLs failed to show significance with all analytical methods and should be taken as indicative rather than conclusive. Regions on LGs 1 and 4 were identified as significant by SMR at $p<0.05$ in the 1998 harvest dataset. The maximum LOD values for the LG1-located QTL were 1.93 based on SIM and 2.35 based on CIM, although the empirical threshold for CIM was 2.91, while for the LG4-located QTL the maximum LOD values were 1.3 based on SIM and 3.7 based on CIM. For the IVVDMD QTL from the 1999 harvest and the LG1/LG3-located QTLs from the nursery-grown spring harvest from 2002, there were significant associations identified by SMR and SIM, but CIM failed to identify a maximum LOD value above the empirically-set threshold.

Coincident QTLs were identified on LG3 from the datasets of the glasshouse and nursery-grown spring harvests in 2002 and the 1999 harvest. All additive effects were positive with maximum LOD values ranging from 2.02 to 2.63 explaining 10.7 to 17.2% of the observed phenotypic variance, depending on the analytical method utilised and the experimental dataset (Table 1). LG7 also contained coincident QTLs from both of the spring 2002 harvests. All additive effects were again positive, with values ranging from 2.17 to 3.07 and explaining 10.7 to 17.2% of the observed phenotypic variance, depending on the experimental dataset and analytical method. Individual QTLs were detected on LGs 1 and 4 for the 1998 and 1999 harvests.

Neutral detergent fibre (NDF)

A total of 13 QTLs for NDF were detected from each of the experimental datasets. Nine of the QTLs failed to show significance with all analytical methods. The 6 QTLs on LGs 2 and 5 were not detected by SMR (with the exception of a single marker-trait association identified on LG5 from the 1998 harvest data) or SIM. However, CIM identified these QTL groups in close repulsion linkage. The two QTLs identified on LG5 were concurrently

detected from the 1998 harvest and summer 2002 harvest datasets, with linkage phase consistent between the two datasets. Coincident QTLs were also identified on LGs 3 and 7 from the 1999 harvest and both of the spring 2002 harvests. The coincident QTLs on LG7 displayed significant marker and trait association through SMR. However, the maximum LOD scores from SIM were close to 2.0, and the maximum LOD values under CIM for both experimental datasets were c. 2.5, below the empirically set LOD threshold of approximately 2.6 (Table 1). QTLs were also identified on LGs 1 and 4 through significant marker-trait association, although the maximum LOD scores for SMR and SIM were below the threshold value (c. 2.0), and CIM also failed to identify significant regions. These QTLs should consequently be regarded as only indicative and treated with caution.

Estimated metabolisable energy (EstME)

A total of 8 QTLs for EstME were detected from 4 of the experimental datasets. Single QTLs on LGs 3 and 7 were identified as significant with all detection methods from analysis of each of the 2002 spring harvest datasets. The exception is the LG3-located QTL from the nursery-grown spring harvest from 2002, which was not significantly identified by CIM. In addition, an indicative coincident QTL was identified on LG3 from analysis of the 1999 harvest dataset with significant marker and trait association ($p<0.01$), although maximum LOD values of 1.9 for SIM and 2.3 (with threshold value of 2.9) for CIM were observed. The coincidence of this QTL with those detected from other datasets gives enhanced credence to a genuine effect associated with the relevant region. Individual QTLs were also detected from the 1998 and nursery-grown spring 2002 harvests that were not otherwise identified. The 1998 harvest data set identified QTLs on LGs 1 and 4 that showed significant marker-trait association ($p<0.05$), but SIM identified maximal LOD values of only c. 1.2. For the QTL on LG4, CIM identified a region of significance, but for the QTL on LG1 CIM revealed a maximum LOD value of 2.3 with an empirical threshold of 2.7. The region on LG1 has provided equivocal data for genetic control.

Water soluble carbohydrate (WSC)

A total of 6 QTLs for WSC were detected from datasets of the 1998, 1999 and glasshouse-grown spring 2002 harvests. For two of the QTLs identified on LG5 from the 1998 harvest dataset, only limited supporting evidence was provided by SMR and SIM. However, the two QTLs were identified by CIM as linked in repulsion with additive effects of similar but opposing magnitude (2.56 and -2.41 respectively). The 1999 harvest data set identified QTLs on LGs1 and 7, with markers significantly associated with the trait data ($p<0.01$), but the maximum LOD values detected by SIM were only 1.48 and 1.97 respectively. CIM identified the LG1 QTL as being significant (maximum LOD value = 2.61 with a threshold of 2.58). However, for the QTL on LG7 the LOD value was maximal at 2.39, with a threshold value of 2.58. The 1998 experimental dataset also identified significant marker-trait association ($p<0.01$) with SIM maximal at a LOD value of 1.81, but significant effects were identified with CIM (maximum LOD = 3.18 with a threshold of 2.93). None of the QTLs were detected in coincident locations.

Co-location of herbage quality QTLs and lignin biosynthetic gene loci

Figure 2

Full-length cDNAs for the LpCCR1, LpOMT1 and LpCAD2 lignin biosynthetic genes (Heath et al. 1998; Lynch et al. 2002; McInnes et al. 2002) were used to detect RFLP in the p150/112 progeny set. Single polymorphic loci were detected for LpOMT1 using the enzyme DraI and for LpCCR1 using the enzyme EcoRI, while three polymorphic loci were detected for LpCAD2 using the enzyme EcoRI. The segregating loci were mapped within the framework of the ILGI reference map dataset (Jones et al. 2002a), detecting four loci designated xlpomt1, xlpccr1, xlpcad2.1 and xlpcad2.3, respectively. The second polymorphic RFLP locus detected by LpCAD2 (on the basis of descending molecular size) failed to group with any of the 7 LGs. The xlpcad2.3 locus was located in the lower central region of LG2. By contrast, the xlpcad2.1, xlpccr1 and xlpomt1 loci were

closely linked within an interval of 0.9 cM on LG7, adjacent to the heterologous RFLP loci xpsr154 and xpsr690. The addition of genomic DNA-derived SSR markers to this framework indicates that the xlpssrk14f07, xlpssrk10h-5 and xlpssrrk14b01 loci are also located within this region (Figure 2), which coincides with the herbage quality QTL cluster.

The fructosyltransferase homologue-encoding LpFT1 and LpFT2 genes were also assigned to the p150/112 map, detecting single genetic loci in the upper distal regions of LGs 7 and 6, respectively (Lidgett et al. 2002; Johnson et al. 2003). However, none of the WSC QTLs identified in this study co-locate with these gene loci.

Comparative genomics of lignin biosynthetic genes in perennial ryegrass and wheat

Table 2

Wheat ESTs showing significant nucleotide similarity to annotated lignin biosynthetic genes from perennial ryegrass and from other plant species were identified through annotation criteria (Table 2). Significant matches to each of the perennial ryegrass genes detecting LG7 loci were observed, and two of the selected wheat ESTs (BE426229 and BE498785) showed the most significant TBLASTX results with the LpCAD2 and LpOMT3 genes respectively. LpOMT1 and LpOMT3 are very closely related at the nucleotide level (Heath et al. 1998). The most significant matches for the other wheat ESTs were with annotated lignin biosynthesis genes from other species, either exclusively, or in addition to less significant results with perennial ryegrass genes.

Figure 3

The chromosomal locations of the wheat ESTs that are ortholoci of known OMT, CCR and CAD genes were determined based on the wheat deletion bin map (Figure 3). ESTs related to each of the LpOMT1, LpCCR1 and LpCAD2 genes are located within adjacent deletion bins at the distal end of chromosome 7DL. Putative ortholoci were also located in distal locations on the other homoeologous group 7 chromosomes (LpCCR1 and LpCAD2 on 7AL; LpOMT1 and LpCAD2 on 7BL). Each of

the perennial ryegrass genes also shows high sequence similarity to Oryza sativa ssp. japonica rice BAC clones assigned to chromosome 8 by BLASTN analysis (LpOMT1: E = 3 x 10^{-144}; LpCCR1: E = 8 x E^{-145}; LpCAD2: E = 1.3 x 10^{-150}: J.W. Forster, unpublished data), and the putative rice ortholocus of LpCCR1 has been attributed to this region (McInnes et al. 2002). Rice chromosome 8 is the syntenic counterpart of the relevant regions of the perennial LG7 and Triticeae homoeologous 7L chromosomes (Jones et al. 2002a).

The homoeologous group 3 chromosomes also contained putative ortholoci for each perennial ryegrass gene in distal bins (LpOMT1, LpCCR1 and LpCAD2-related loci on 3AL and 3DL; LpCCR1 and LpCAD2-related loci on 3BL). In addition, ESTs related to two of the three gene classes were located to the distal regions of 2BS, 2DS, 6AL and 6DL, and ESTs related to single gene classes were mapped to the distal regions of 2AL and 6BL, as well as the interstitial regions of 5AL, 5BL and 5DL.

The distal regions of the wheat group 3L and 7L chromosomes are the syntenic counterparts of the corresponding regions of perennial ryegrass LGs 3 and 7, in which herbage quality QTL clusters are located. Although the perennial ryegrass lignin biosynthetic genes did not detect polymorphic RFLP loci on LG3, the location of OMT, CAD and CCR-related wheat ESTs on 3L suggests that other members of these gene families, that were not detected by RFLP analysis in the reference population, may be located on this linkage group.

Discussion

Genetic dissection of herbage quality traits

A total of 42 QTLs for herbage quality traits in perennial ryegrass were detected from the 6 experimental datasets. Groups of coincident QTLs were identified on LGs 3, 5 and 7 and can be rationalised into 8-9 key target regions for potential breeding applications. The use of various forms of QTL analysis such as SMR, SIM and CIM is critical for the comprehensive dissection of these datasets. Judicious comparative analysis of the overall dataset by the differing approaches permitted the identification of both unequivocal QTLs that are detected with high significance with all methods, and indicative QTLs which should be treated with caution. The IM methods were largely in agreement over QTL identification. However, in several instances conflicting results have been obtained for the presence of effective genomic regions, such as the QTLs for IVVDMD on LGs 1 and 3 from the nursery-grown spring harvest in 2002 and the QTL for EstME LG4 from the 1998 harvest. The data summarised in Table 1 consequently represent the QTLs that are detected by all three analytical methods, those that are detected by at least one method, and a small number of putative QTLs that fail significance with all three methods, but closely approach the significance level with at least one form of analysis.

Substantial groups of coincident QTLs were located on LGs 3 and 7. The region on LG3 was associated with variation for all measured traits across various experimental datasets. For each sampling experiment, with the exception of the summer harvest data from 2002, the LG3 region was identified as significant for at least one trait. A major genomic region associated with herbage quality variation is defined by this analysis, providing a potential target for marker-assisted selection (MAS). Similarly, the cluster of coincident QTL locations on LG7 represents each of the traits apart from CP. The majority of QTLs in this region were contributed by the two spring harvests in 2002, but the WSC QTL from the 1999 dataset is also located in this region.

The two spring harvests from 2002 obtained consistent comparable QTL locations for different traits in the regions of LG3 and LG7. A comparison of the data from these two harvests provides evidence for stability of genetic control between glasshouse-grown and nursery-grown samples. The observed co-locations suggest that the QTLs detected by NIRS analysis under controlled growth conditions may be sufficiently stable to permit MAS for field-expressed performance. At the same time, variation is observed in a number of genomic locations for coincidence of QTLs for the same trait measured in experiments varying by season, location and year. This provides preliminary evidence for QTL x E (environment) variation, which has been observed in a number of detailed studies (Paterson et al. 1991; Lu et al. 1996; Yan et al. 1999; Yadav et al. 2003), although the environmental parameters contributing to the effect are in many cases unknown (Paterson et al. 2003). The presence of QTL x E interactions for nutritive value traits is consistent with the known effects of environmental factors such as reproductive development in grasses (Oram et al. 1974; Tyler and Hayward 1982). However, genotypes of grass species have been identified that consistently exhibit high nutritive value across a range of environments and seasons (Casler 2001; Smith et al. 2004). The relative stability of QTL effects associated with the LG3 and LG7-located clusters provide the best option to overcome problems associated with QTL x E in MAS applications derived from the current study.

Although for the NDF and WSC traits no significant correlation was detected between marker and trait data using SMR, and SIM analysis did not identify significant QTLs on LG5, CIM detected two QTLs in repulsion on this LG for each trait from three of the experimental datasets. Significant QTLs were identified for NDF from the 1998 and the summer 2002 harvests, and in addition WSC QTLs were detected from the 1998 harvest. The additive effects of the QTLs were negative and positive respectively for NDF, and positive and negative respectively for WSC. A similar pattern was observed for the QTLs for these traits on LG3, with the additive effect opposed in direction between NDF and all other measured traits at each location. These relationships are predictable due to the observed negative correlation between phenotypic variation for NDF and

for the other traits. The digestibility of the NDF fraction of forage varies between 100% (mesophyll) and 0% (xylem) in some plants (Akin 1989), with the absolute value influenced by plant maturity in ryegrasses (Armstrong et al. 1992), and the digestibility of the soluble component of herbage is usually 100%. In consequence, any increase in the NDF concentration of herbage is likely to be associated with a concomitant decrease in IVVDMD. Conversely, as forage dry matter is the sum of NDF and neutral detergent solubles (such as CP and WSC), any increase in concentration of the soluble components of herbage that is not merely associated with a change in the partitioning of dry matter between these components must lead to a reduction in NDF and a corresponding increase in IVVDMD**.**

Reproductive development was anticipated to influence the expression of phenotypic variation for CP concentration (and potentially other traits such as NDF, IVVDMD and WSC) in the mapping population. This was indicated by the change in direction of effect of the additive genetic component betweeen QTLs for CP on LG3 for the spring harvests in 2002 and the summer harvests in 1998 and 2001, respectively. Seasonal variation for CP concentration is expected for ryegrass species due to changes in plant nitrogen content associated with alterations in the ratio of stems, leaf sheaths and lamina. These structures have contrasting nitrogen content, and hence CP concentrations (Armstrong et al*.* 1992).

Candidate gene-QTL co-location

The coincident herbage quality QTLs on LG7 were assigned to a region of c. 28 cM maximum length based on a decline of 2 LOD units from maximum values through CIM analysis. This region is extensive at the molecular level, given an average relationship between genome size (c. 1.6 x 10^9 bp haploid content: Hutchinson et al. 1979; Seal and Rees 1982) and map distance (814 cM: Jones et al. 2002b) of c. 2 Mb/cM. However, within this region close linkage is observed between RFLP loci detected by cDNAs corresponding to three of the major classes of enzymes in the pathway to monolignol biosynthesis: caffeic acid-*O*-methyltransferase (OMT), cinnamoyl CoA-reductase (CCR) and cinnamyl alcohol

dehydrogenase (CAD). The maximum LOD locations for a number of the QTLs coincides with the position of the lignin biosynthesis gene cluster. The observation of co-location between these candidate gene loci and a major QTL cluster suggests that allelic variation either in coding sequences or regulatory regions (Paran and Zamir 2003) may contribute to the phenotypic variation for target traits. Confirmation of this hypothesis will entail more extensive analysis including association studies through linkage disequilibrium (LD) mapping (Thornsberry et al. 2001; Rafalski 2002; Gaut and Long 2003; Flint-Garcia et al. 2003), in concert with the production of phenocopies through transgenic modification such as gene silencing (Vance and Vaucheret 2001). In this context, we are performing single nucleotide polymorphism (SNP) development for the full-length LpCCR1 and LpCAD2 genes, and antisense transgenic plants have been generated for each of the LpOMT1, LpCCR1 genes. The successful validation of candidate gene-based markers for components of herbage digestibility would permit genotypic selection on the basis of superior allele content (Sorrells and Wilson 1997) for pasture grass breeding (Forster et al. 2004).

Comparative genomics of lignin biosynthetic genes

The identification of substantial macrosynteny between the genomes of perennial ryegrass and the Triticeae cereals (Jones et al. 2002a) provides the opportunity for comparative genomics analysis of shared traits and metabolic processes, including herbage digestibility and lignification. These relationships are consistent with the comparative location of LpOMT1, LpCCR1 and LpCAD2-detected RFLP loci in the lower central region of perennial ryegrass LG7 and the assignment of related wheat ESTs to a distal deletion bin on 7DL, in a region of predicted conserved synteny. Wheat ESTs related to LpOMT1, LpCCR1 and LpCAD2 also mapped to the distal ends of wheat chromosomes 3AL and 3DL, suggesting that the locations of these genes in the wheat genome may arise from ancient duplication events, with similar linear orders. However, the wheat ESTs related to LpCAD2 and LpCCR1 differ between the group 3 and group 7 chromosomes, possibly due to independent gene

divergence following duplication. Such duplication-gene divergence evolutionary events have also been observed in alignments between rice chromosome 1 and wheat 3S (Francki et al. 2004). The duplication–divergence hypothesis is further supported by the assignment of distinct OMT-related wheat ESTs to deletion bins on each of homeologous groups 2, 3, 6 and 7. This suggests that each lignin biosynthesis gene class may be represented in wheat by multiple diverged copies, and that members of each class may be located in close association at each bin location, but have not yet been mapped. A preliminary TBLASTX comparison of the perennial ryegrass gene sequences against the wheat EST database has identified other ESTs with significant sequence similarity that have not yet been located by deletion bin mapping (data not shown). Subsequent mapping of these ESTs may provide direct evidence for segmental duplications of a lignin biosynthesis gene cluster during Poaceae evolution, with current representatives on wheat groups 2, 3, 5, 6 and 7.

Due to the relatively close phylogenetic relationship between the Triticeae and Poaceae grasses, perennial ryegrass may share a segmental duplication pattern. The LpOMT1 and LpCAD2 cDNAs detected small multigene families in genomic Southern hybridisation experiments (Heath et al. 1998; Lynch et al. 2002), although LpCCR1 revealed a lower genomic complexity. As only a small proportion of the genomic loci revealed RFLP in the p150/112 population, it is possible that loci other than those detected on LG7 could be detected in other pedigrees, and that paralogous gene variation on LG3 may contribute to the QTL effects associated with this LG. The development of locus-specific SNP markers for the lignin biosynthesis genes will permit specific map assignment and confirm whether the existing cDNAs are derived from LG7-located loci, or other related genomic locations.

Comparative analysis of lignin biosynthesis genes provides the opportunity for detection of orthologous QTLs between species, with the potential to target chromosomal regions in wheat and its relatives for lignin-related traits, such as cereal residue digestibility. In this context, recent research has identified a major QTL for the traits of solid stem and sawfly resistance in the distal region of wheat 3BL, in a region co-

localising with the LpCAD2 orthologous (Cook et al. 2004). Conversely, advances in physical mapping of wheat ESTs provides the basis for orthologous identification and exploitation in perennial ryegrass.

Comparative genetic analysis may also be extended to more distant relatives of the Poeae grasses within the Poaceae family that are used as forage species, such as maize (Zea mays L.). Breeding improvement for high digestibility in forage maize has been defined as an important objective for animal nutrition (Lundvall et al. 1994). Fibre and lignin content traits such as NDF, acid detergent fibre (ADF) and acid detergent lignin (ADL) were measured by NIRS in a recombinant inbred line (RIL) maize mapping family (Cardinal et al. 2003). ADF is related to EstME, and ADL is negatively correlated with IVVDMD. Multiple QTLs for each trait were detected, with substantial clustering on chromosomes 1, 2, 3, 5, 6, 7, 8, 9 and 10. Coincident locations were observed with QTLs detected in a previous study (Lübberstedt et al. 1997). The conserved synteny relationships between the genomes of perennial ryegrass and maize are not as well understood as for the Triticeae cereals. However, LGs 3F and 7F in meadow fescue (Festuca pratensis Huds.), which are largely colinear with their perennial ryegrass counterparts (Alm et al. 2003), correspond to regions of maize chromosomes 3/8 (3F) and 6/9, 1/5 (7F) respectively. Each of these chromosomes contains QTL clusters for putative orthologous traits to those described in the present study. In addition, several maize QTLs coincide with the location of bm (brown mid-rib) mutant loci associated with lignin biosynthesis, such as the CAD-related bm1 locus, which maps to chromosome 5 (Baucher et al. 1998).

Breeding implications

The results of the marker-trait QTL association studies described in this study provide efficient and valuable selection mechanisms for either components of digestibility that are expressed throughout the growing season, or traits associated with the post-reproductive decline in digestibility. This targeted approach to improving nutritive value in ryegrass species will prevent the need for detailed and logistically complex sampling strategies that seek to negate the effects of environmental

variation. Important additional benefits will be obtained through the breeding of cultivars to improve the late spring and early summer seasonal deficiencies that limit forage quality in Australian pasture systems.

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Table Legends

Table 1

Summary of QTL analysis data for NIRS-calibrated herbage quality traits in the p150/112 reference mapping population. QTL identification was performed using the QTL Cartographer software and SMR, SIM and CIM analyses were performed. QTLs identified as significant with all three analytical methods are shaded in grey. The criteria for inclusion of other QTLs were either significant detection with at least one of the analytical methods, or the observation of maximum LOD values close to, but not exceeding, the threshold values.

Table 2

Summary of sequence annotation data for lignin biosynthesis gene-related ESTs of hexaploid wheat (Triticum aestivum L.) compared to putative orthologous sequences from L. perenne and other species. Alignments represent percentage amino acid identity over the length of the EST (in nucleotides). E values for TBLASTX hits are shown in parentheses.

Figure Legends

Figure 1

Location of QTLs for NIRS-calibrated herbage quality traits on the p150/112 reference genetic map of perennial ryegrass. Nomenclature of genomic DNA-derived SSR (LPSSR) loci, AFLP loci and heterologous RFLP loci is as described by Jones et al. (2002a,b). QTL nomenclature is adapted from McCouch et al. (1997) in the form q-TRAIT-season-location-year, with details as described in footnote 1 to Table 1. All QTL locations were derived from CIM analysis. All putative QTLs described in Table 1 are shown, with the exception of the equivocal loci qIVVDMD-98, qNDF-98, qEstME-98 and qWSC-98. Bars and lines represent 1 and 2 LOD unit drops from the maximum likelihood value.

Figure 2

Detailed genetic map of the lignin biosynthesis gene cluster on perennial ryegrass LG7. The xlpcad2.1, xlpccr1 and xlpomt1 loci were mapped within the framework of the AFLP and heterologous RFLP-based map of Jones et al. (2002a). Genomic DNA-derived SSR (xlpssr) loci (Jones et al. 2002b) are shown as accessory markers within the target region.

Figure 3

Location of lignin biosynthesis gene-related wheat ESTs to deletion bins of hexaploid wheat. The BE and BF prefixes denote EST origin, and the matching gene class is shown in parenthesis following the EST number.

Table 1

			SMR	SIM				CIM based on 1000 simulations			
	Trait[1]	LG	P<0.01	Max LOD score	Position	a^2	R^3	LOD threshold	Max LOD score	Position	a^2	R^3
CP	qCP-su-gh-02	1	21.4-25.6 (29.4-43.9 p<0.05)	1.63	23.41	1.715	0.093	2.79	3.02	23.41	2.646	0.143
	qCP-su-gh-01	2	41-60.2	2.28	47.61	-1.146	0.159	2.83	3.19	47.61	-1.173	0.151
	qCP-sp-gh-02	3	68.9-89.7 (83.7 0.05)	2.51	70.90	-1.746	0.108	2.67	3.29	68.91	-1.920	0.121
	qCP-su-gh-01	3	(83.7-89.7 p<0.05)	1.35	87.11	0.877	0.097	2.83	2.88	87.11	1.081	0.140
	qCP-98	3	89.7-116.8	1.45	95.70	0.836	0.106	2.92	2.52	99.71	1.042	0.153
	qCP-98	4	78.6-87.2 (90.7 p<0.05) 95.7-116.7	3.06	107.11	1.124	0.193	2.92	3.62	103.11	0.971	0.136
	qCP-99	5	36.1-42.7; 52.4	1.67	52.41	2.369	0.065	2.78	2.02	46.61	2.541	0.071
IVVDMD	qIVVDMD-98	1	0-13.8 p<0.05	1.31	6.01	-1.067	0.087	2.91	2.35	6.11	-1.285	0.116
	qIVVDMD-sp-gh-02	3	68.9-116.8	3.31	83.71	2.549	0.150	2.82	4.33	83.71	2.596	0.153
	qIVVDMD-sp-nu-02	1	21.4-29.4 (34.1 p<0.05) 40.5-53.9	2.23	43.91	2.435	0.107	2.80	0.78	43.91	1.572	0.030
	qIVVDMD-99	3	55.5-72.5	2.15	72.51	2.269	0.088	2.79	2.53	72.51	2.316	0.091
	qIVVDMD-sp-nu-02	3	31.5-72.5	2.58	36.31	2.632	0.114	2.80	1.22	36.31	2.022	0.044
	qIVVDMD-98	4	(52.1-72.3 p<0.05)	1.30	65.41	1.051	0.079	2.91	3.69	60.61	1.704	0.171
	qIVVDMD-sp-nu-02	7	60.3-120.6	2.45	110.51	3.078	0.172	2.80	3.02	65.91	2.534	0.113
	qIVVDMD-sp-gh-02	7	65.9-98.5	2.30	73.01	2.168	0.107	2.82	3.19	71.01	2.286	0.115
NDF	qNDF-sp-nu-02	1	0; 13.8-29.4; 43.9-53.9	1.93	43.91	-1.730	0.089	2.63	0.66	43.91	-0.942	0.020
	qNDF-sp-nu-02	2	-	0.34	77.81	0.710	0.015	2.63	2.70	116.81	2.814	0.110
	qNDF-sp-nu-02	2	-	0.82	131.11	-1.093	0.036	2.63	3.55	131.11	-4.053	0.118
	qNDF-99	3	68.9-72.5	2.17	72.51	-2.456	0.090	2.71	3.42	72.51	-3.129	0.133
	qNDF-sp-gh-02	3	72.5-116.8	2.93	83.71	-1.896	0.134	2.67	3.78	83.71	-1.869	0.130
	qNDF-sp-nu-02	3	22.6-72.5	2.99	36.31	-2.163	0.128	2.63	3.16	55.51	-1.902	0.100
	qNDF-sp-gh-01	4	54.3-58.6	1.90	54.31	-1.647	0.128	2.75	1.86	51.51	-2.299	0.101
	qNDF-98	5	0	0.24	6.00	0.596	0.013	2.83	4.08	0.00	-2.579	0.214
	qNDF-98	5	-	0.42	30.41	-0.703	0.019	2.83	2.03	63.31	1.692	0.090
	qNDF-su-gh-02	5	-	1.20	44.61	-1.637	0.067	2.83	3.38	51.91	-3.687	0.170
	qNDF-su-gh-02	5	-	0.17	82.71	0.673	0.011	2.83	2.87	65.31	4.570	0.224
	qNDF-sp-gh-02	7	65.9-98.5	2.00	73.01	-1.580	0.092	2.67	2.49	69.01	-1.485	0.081
	qNDF-sp-nu-02	7	88.5-98.5	1.95	88.51	-1.695	0.086	2.63	2.48	35.91	-1.705	0.087

			SMR	IM				CIM based on 1000 simulations				
	Trait[1]	LG	P<0.01	Max LOD score	Position	a^2	R^3	LOD threshold	Max LOD score	Position	a^2	R^3
EstME	qEstME-98	1	(0-13.8 p<0.05)	1.29	6.01	-0.180	0.086	2.77	2.34	6.11	-0.218	0.116
	qEstME-sp-nu-02	1	21.4-53.9	2.23	43.91	0.388	0.107	2.78	0.78	43.91	0.251	0.030
	qEstME-99	3	55.5; 68.9-72.5	1.93	72.51	0.345	0.080	2.91	2.30	72.51	0.353	0.083
	qEstME-sp-gh-02	3	68.9-116.8	3.32	83.71	0.407	0.150	2.81	4.34	83.71	0.415	0.153
	qEstME-sp-nu-02	3	31.5-44.8; 50.4-72.5	2.58	36.31	0.420	0.114	2.78	1.22	36.31	0.313	0.042
	qEstME-98	4	(52.1-72.3 p<0.05)	1.24	62.61	0.171	0.072	2.77	3.71	60.61	0.290	0.172
	qEstME-sp-gh-02	7	65.9-98.5	2.28	73.01	0.345	0.107	2.81	3.17	71.01	0.370	0.113
	qEstME-sp-nu-02	7	60.3-75.5; 88.5-120.6	2.43	110.51	0.490	0.171	2.78	3.01	65.91	0.404	0.113
WSC	qWSC-99	1	84.1	1.48	27.61	1.890	0.082	2.58	2.62	17.81	-4.963	0.089
	qWSC-98	2	56.6-65.5	1.81	58.61	1.799	0.106	2.93	3.19	58.61	2.128	0.141
	qWSC-sp-gh-02	3	55.5; 68.9-116.8	4.45	72.51	5.243	0.186	2.76	4.52	87.11	5.210	0.181
	qWSC-98	5	30.4	1.47	30.41	1.550	0.080	2.93	3.48	30.41	2.557	0.141
	qWSC-98	5	-	0.52	10.00	-0.174	0.027	2.93	2.48	60.41	-2.407	0.126
	qWSC-99	7	120.6	1.97	116.51	4.976	0.116	2.58	2.39	120.51	4.944	0.110

[1]QTL nomenclature adapted from McCouch et al. (1997) in the form q-TRAIT-season-location-year. The suffixes relate to experimental datasets as follows: -sp-gh-02 = glasshouse-grown material in spring 2002; - sp-nu-02 = nursery-grown material in spring 2002; -su-gh-01 = glasshouse-grown material in summer 2001; - su-gh-02 = glasshouse-grown material in summer 2002; -98 = glasshouse –grown material from 1998; -99 = glasshouse –grown material from 1999.

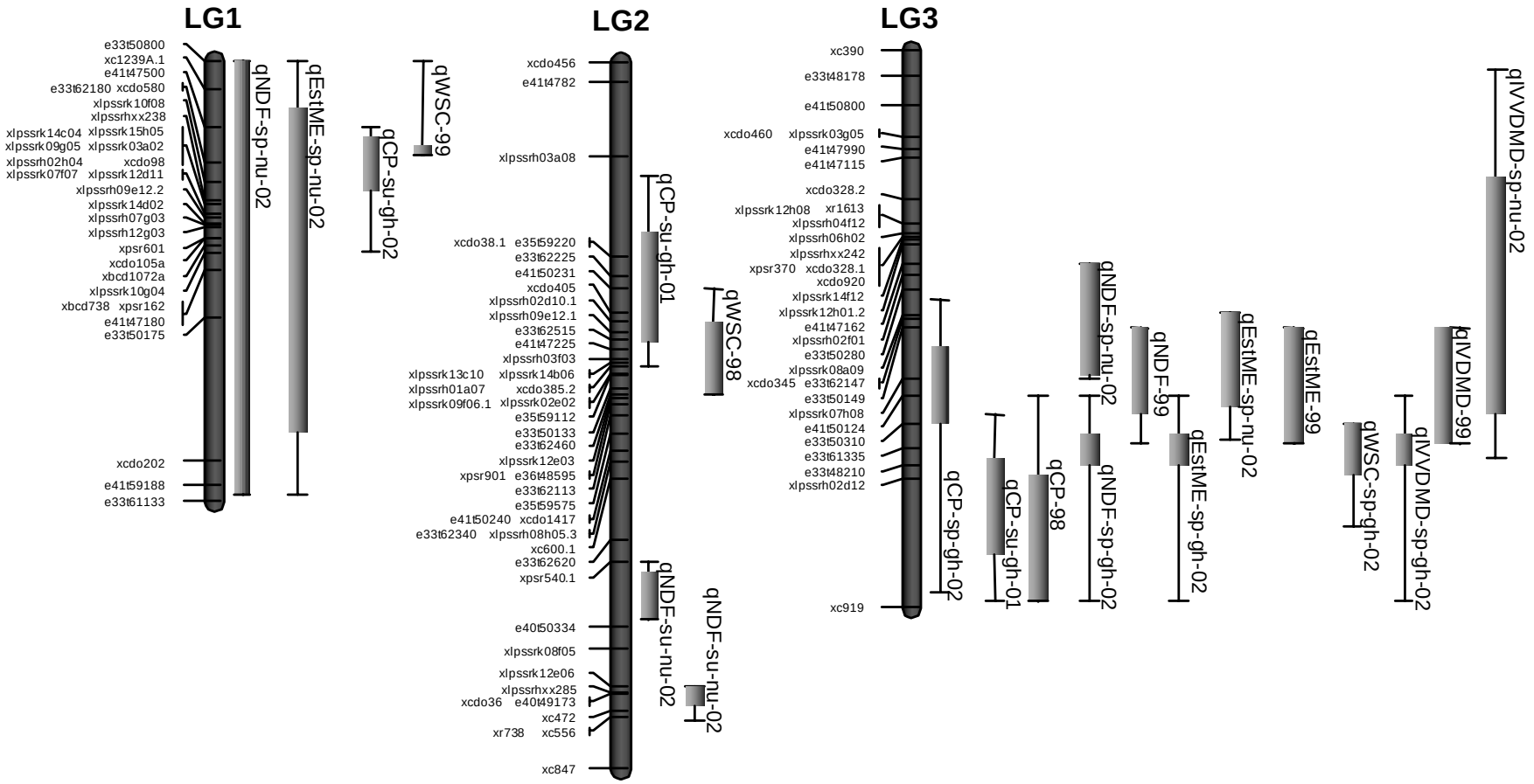
[2]Additive effect of substituting alternative alleles at marker locus.

[3] Proportion of variance explained by QTL.

Table 2

Wheat EST	EST length	TBLASTX match	Alignment	Best TBLASTX match (annotated)	Alignment
BF482769	556	L. perenne OMT1 (AF033538)	81%: 3-554 (1e-102)	T. aestivum COMT1 (AY226581)	85%: 3-554 (1e-105)
BE426229	605	L. perenne OMT3 (AF033540)	60%: 4-294 (1e-70); 60%: 277-603 (1e-70);	L. perenne OMT3 (AF033540)	60%: 4-294 (1e-70); 60%: 277-603 (1e-70);
BE498785	676	L. perenne CAD2 (AF472592)	75%: 61-393 (9e-53); 81%: 392-676 (4e-48)	L. perenne CAD2 (AF472592)	75%: 61-393 (9e-53); 81%: 392-676 (4e-48)
BE404596	566	No L. perenne hits	No L. perenne hits	S. cereale OMT (AY177404)	67%: 90-455 (2e-51)
BE406497	173	L. perenne CCR1 (AY061888)	49%: 8-172	A. thaliana CCR (AY093143)	51%: 8-172 (2e-12)
BF293181	534	L. perenne CCR1 (AF278698)	37%: 154-534 (9e-23)	A. thaliana CCR (AY093143)	49%: 22-378 (5e-35); 46%: 434-505 (5e-35)
BE443397	600	L. perenne CAD2 (AF472592)	59%: 9-320 (2e-65); 51%: 335-598 (2e-65); 41%: 600-884 (6e-8)	A. thaliana ADH (AY288079)	71%: 6-326 (3e-91); 75%: 335-598 (3e-91)
BF293156	566	No L. perenne hits	No L. perenne hits	Z. mays OMT (MZEOMT)	51%: 3-215 (4e-34); 40%: 309-383 (4e-34); 39%: 396-563 (4e-34)
BE443747	571	No L. perenne hits	No L. perenne hits	A. thaliana CCR (BT002742)	49%: 11-127 (2e-29); 40%: 245-544 (2e-29);

Figure 1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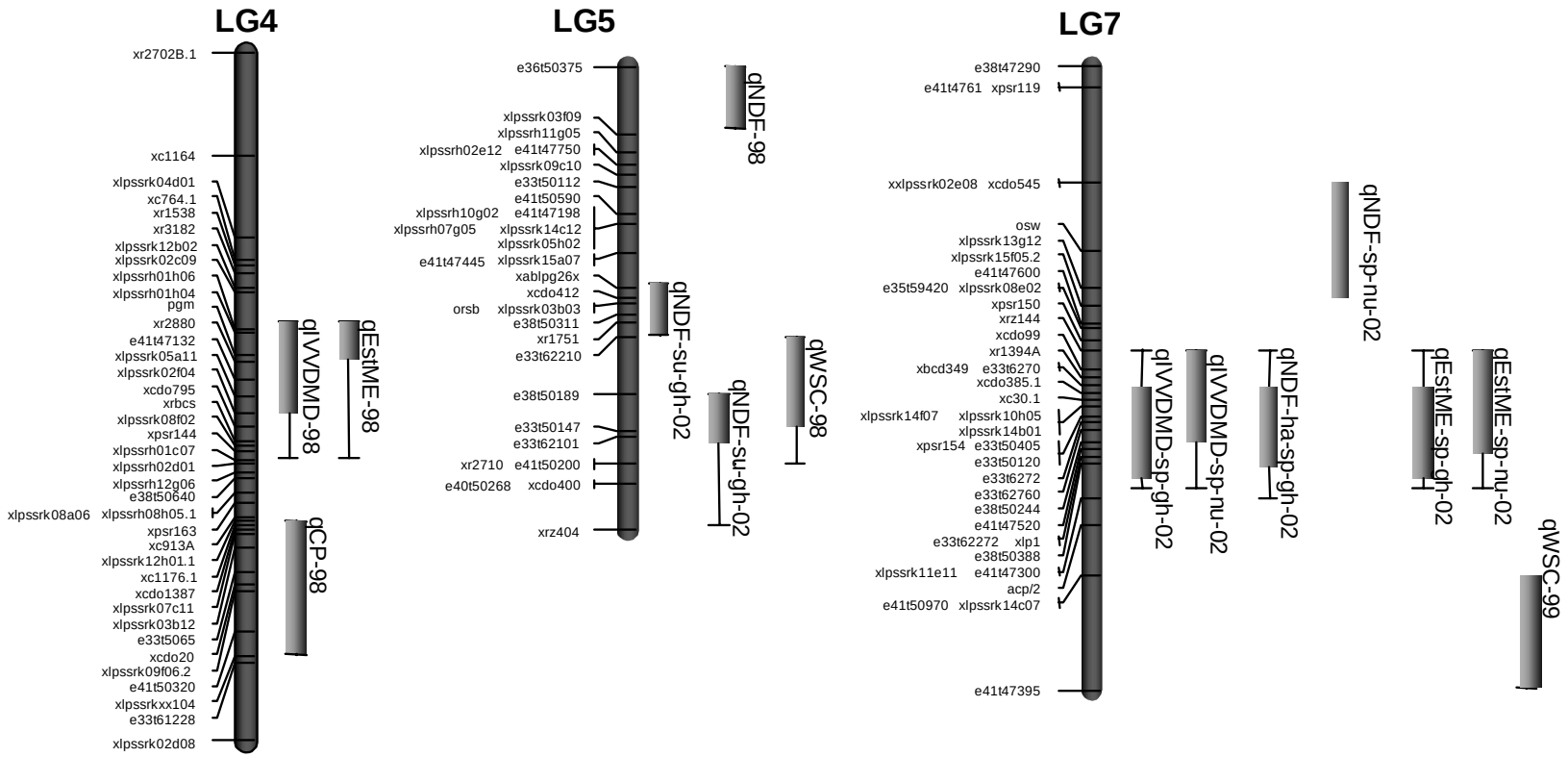
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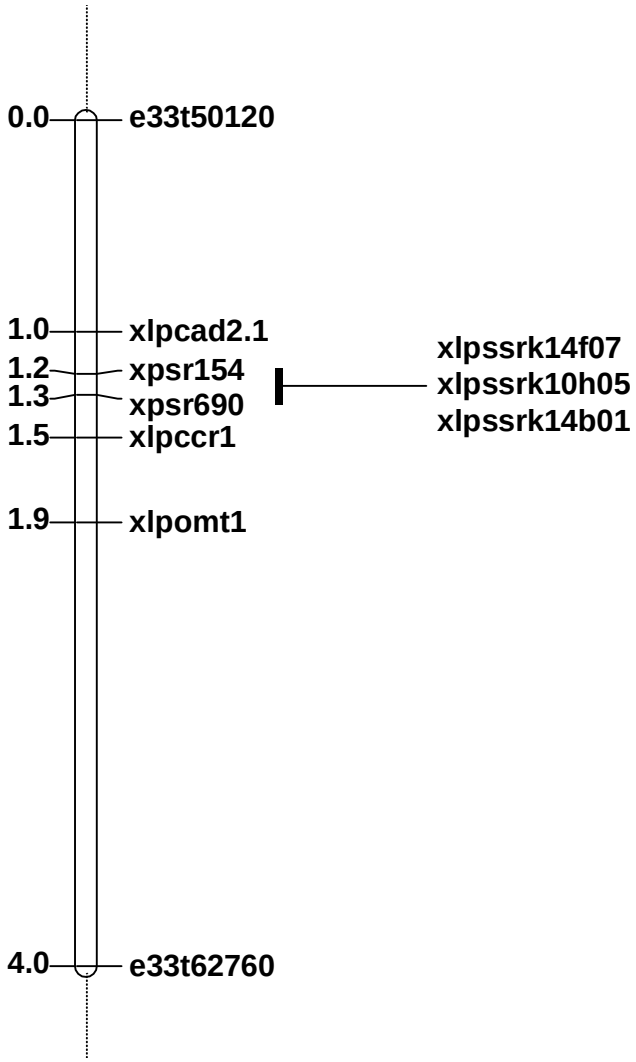
Figure 2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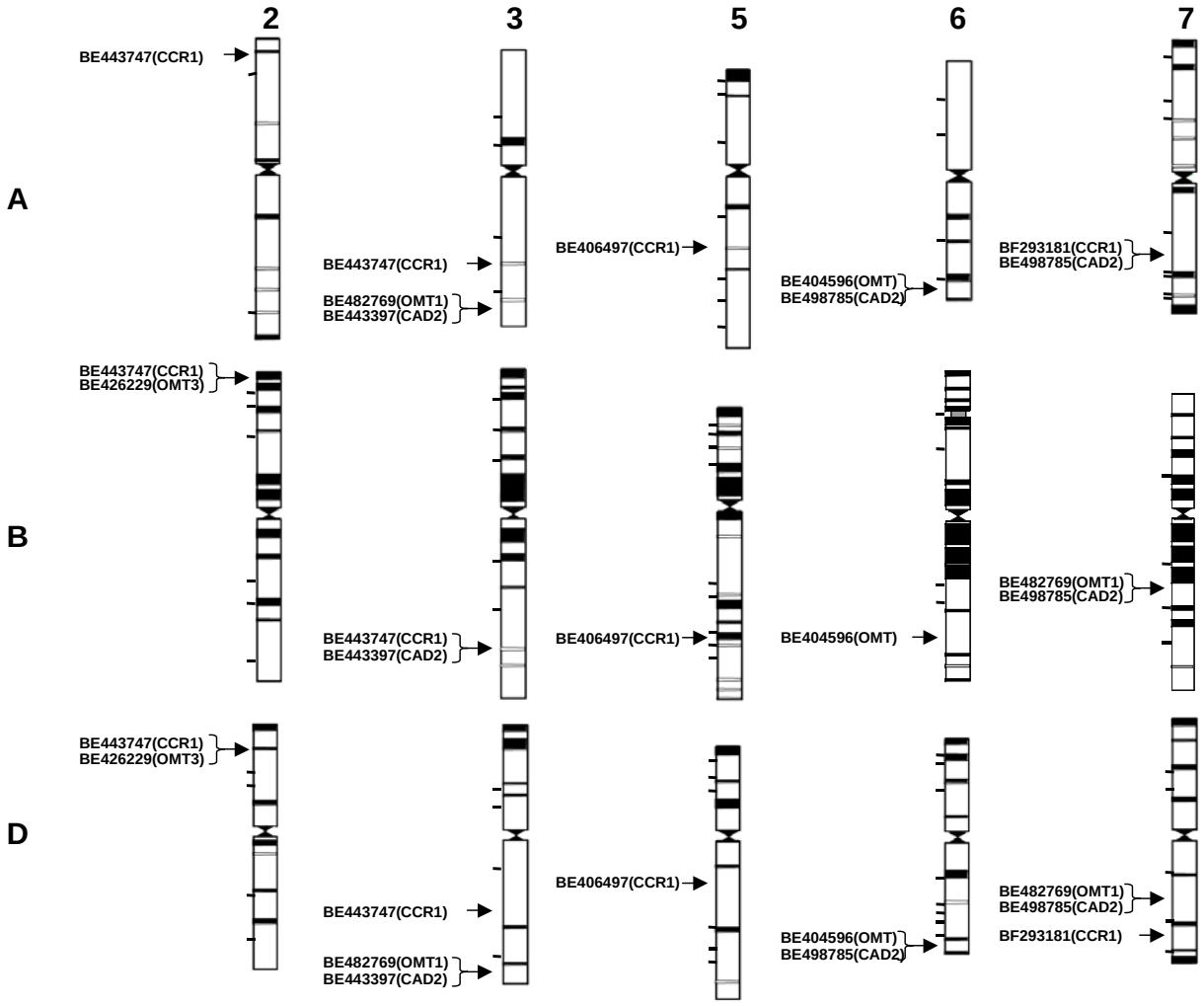
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